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Exploiting novel tailored immunotherapies of type 1 diabetes: Short interfering RNA delivered by cationic liposomes enables efficient down-regulation of variant *PTPN22* gene in T lymphocytes

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Abstract

In autoimmune diseases as Type 1 diabetes, the actual treatment that provides the missing hormones is not able, however, to interrupt the underlining immunological mechanism. Importantly, novel immunotherapies are exploited to protect and rescue the remaining hormone producing cells. Among probable targets of immunotherapy, the C1858T mutation in the *PTPN22* gene, which encodes for the lymphoid tyrosine phosphatase (Lyp) variant R620W, reveals an autoimmunity related pathophysiological role. Our scope was to establish new C1858T *PTPN22* siRNA duplexes delivered by liposomal carriers (lipoplexes) to patients' PBMC. Following lipoplexes treatment, $CD3^+$ and $CD3^-$ immunotypes were efficiently transfected; cell integrity and viability were preserved. Specific target mRNA down-modulation was observed. After T cell receptor stimulation, in lipoplexes-treated PBMC Lyp function was restored by increased release of IL-2 in cultures. Results set-up the stage for ultimate trials in the treatment of autoimmunity based on the specific inhibitory targeting of C1858T *PTPN22* by lipoplexes.

Key words: T1D; Lipoplexes; Immunotherapy; Variant *PTPN22*

Abbreviations: Abs, antibodies; AG, autoimmune gastritis; APS3v, autoimmune polyglandular syndrome Type 3 variant; ATD, autoimmune thyroid disease; CD, celiac disease; CSK, C-terminal Src Kinase; DMPC, dimyristoyl-*sn*-glycero-phosphatidylcholine; GADA, glutamic acid decarboxylase Abs; HET, heterozygous; h, hours; IA-2, second islet antigen; IAA, anti-insulin Abs; IDA, iron-deficiency anemia; IFL, indirect immunofluorescence; IL-2, interleukin 2; IRB, Institutional Review Board; Lyp, lymphoid tyrosine phosphatase; MHC, major histocompatibility complex; NOD, non obese diabetic; O/N, overnight; PCA, parietal cells Abs; *PTPN22*, protein tyrosine phosphatase N22 gene; Rev, reviewed; RT, room temperature; rtq-PCR, quantitative Real-Time PCR; SLE, systemic lupus erythematosus; T1D, Type 1 diabetes; TCR, T cell receptor; Tg, Thyroglobulin; TLR, Toll-like receptor; TPO, thyroperoxidase; Tregs, T regulatory cells; tTGA, transglutaminase; V, vitiligo; vs, versus; WT, wild-type.

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Insulin-dependent diabetes mellitus (Type 1 diabetes, T1D)[1] is an organ-specific autoimmune endocrinopathy, where autoreactive T lymphocytes cause islet β cells destruction. T1D is the third most common metabolic disorder in the world after obesity and thyroid disorders.[2,3] Recent epidemiological studies estimate that the prevalence of autoimmunity and, diabetes in particular, has increased over the past 30-40 years worldwide in children <15 years (0.1/100.000 in China and 40.9 /100.000 in Finland). Clinically, T1D presents with interrelated metabolic, vascular and neuropathic sequelae and, since the disease onset, if it is not promptly treated with insulin, severe clinical manifestations can occur such as ketoacidosis, potential coma and death. Nevertheless, insulin treatment administered in multiple daily injections will never reproduce the physiological circadian rhythm of the molecule. A significant advance beyond the state of art is therefore the effect that any immunotherapeutic intervention may play in halting the pathogenic immunological mechanisms, preserving therefore the residual hormone producing cells.[4] This could avoid the typical 'instability' of the metabolic course of disease that requires adjustments on daily insulin administration and continuous glucose monitoring, thereby preventing or reducing long-term complications.

Several immunotherapeutic approaches have being experimented in T1D. Nonetheless, the majority of trials based either on antigen-specific therapies, targeting of T and B lymphocytes, anti-inflammatories, stem cells or cytokines, did not lead to insulin-independence in diabetic patients.[5] The reason may be related to the contribution of environmental factors, dose, time and extent of treatment but also to disease heterogeneity since a variety of gene variations can be found in T1D[6] (reviewed (rev) in[7]), and thus influence their etiopathogenesis. In this regard, T1D susceptibility is influenced by major histocompatibility complex (MHC) as well as non-MHC genes, i.e. *INS-VNTR,*[8] *CTLA-4,*[9] *SUMO-4*[10] and *PTPN22*.[7]

In the light of the foregoing, the use of 'tailored approaches' of personalized medicine based on 'specific' gene targeted therapies can be envisaged.[11] In this regard, recent interest was focused on the potential pathophysiological role played by the C1858T *PTPN22* (protein tyrosine phosphatase N22 gene) mutation, with Arg (R) 620 toward Trp (W) (R620W) amino acidic change in the encoded protein Lyp (lymphoid tyrosine phosphatase).[11]

In humans, R620W is significantly associated with T1D augmenting the risk of developing the disorder by 2-4 fold.[12] Unraveling mechanistic insights, Lyp performs its inhibitor regulatory role on T cell antigen receptor (TCR) signaling acting in interplay with the C-terminal Src kinase (CSK). The overall Lyp variant effect is still a disputed topic in literature. Most works support a 'gain of function' model considering the variant phosphatase as a more potent regulator of T cell signaling[13] paradoxically leading to diminished lymphocyte activation. R620W Lyp could produce defects in TCR signaling and affect the establishment of immunological tolerance at the thymus level in perinatal age and the breakout of autoreactive T cells (rev in[11]), which would normally be eliminated. We also found defects in the homeostasis of B cells and Toll-like receptor (TLR) 9-mediated response in *PTPN22* C1858T T1D[14] confirming its influence on innate and adaptive immunity (rev in[11]). Recent studies highlighted the R620W Lyp variant activity on regulatory T cells (Tregs) (rev in[11,12]) and macrophages.[15] Knockout mice showed enhanced Tregs thymic selection and peripheral Tregs alterations.[12] On the other hand, in the 'loss of function' model, Lyp degradation produces hyper responsiveness of lymphoid and dendritic cells.[16] Here, the loss of self-tolerance happens putatively earlier in T cell life and afterwards is sustained by auto antigens (rev in[11]). Whichever model is considered to support the pathogenic activity of Lyp R620W, this variant remains a compelling target for 'tailored' treatment by means of its down-modulation/knockdown in T1D and autoimmune polyglandular syndrome Type 3 variant (APS3v) patients since it would in any case restore its normal activity.

We recently provided evidence of the possibility of targeting wild-type *PTPN22* gene through siRNA molecules in liposomes (lipoplexes) to reach lymphocytes. Liposomal formulations were previously used in clinical trials, due to their low toxicity and biodegradability (rev in[11]). Specifically, we employed cationic liposomes[11] to deliver siRNA against wild type *PTPN22* to Jurkat T cells and also to human peripheral blood lymphocytes of normal subjects.[11] Thus in exploiting the feasibility of novel immunotherapeutic targets in T1D, in this work we unravel a similar strategy in order to achieve target down-modulation, of variant *PTPN22* gene, using novel siRNA duplexes vehicled by liposomes (lipoplexes).

Methods

Additional details are found in the Supplementary materials sections (S).

Liposomes synthesis and characterization

Gemini surfactant 2R,3S-2,3-dimethoxy-1,4-bis(N-hexadecyl-N,N-dimethylammonium)- butane dibromide, 2, was prepared as previously reported (S1, Supplementary materials).[11,17]

Circular dichroism spectroscopy and dynamic light scattering analysis

For CD spectra and DLS measurements[11] refer to S2 and S3, Supplementary materials.

siRNA design

Authentic siRNA sequences were designed to specifically target C1858T *PTPN22* gene variant (Rosetta Inpharmatics, Sigma-Aldrich Chemical Co., Saint Louis, MO, US). The siRNA sequences, sense/antisense (s/a) duplexes were different for mRNA target affinity (S1 Table) and did not comprise backbone modifications. We employed the sequence corresponding to the highest affinity siRNA sequence, namely (SNP_T sense 5′-GUAUGGACACCUGAAUCAUdTdT-3′; SNP_T antisense 5′-AUGAUUCAGGUGUCCAUACdTdT-3′), for subsequent experiments (Italian Patent Application number 102018000005182 filed on 9 May 2018) (See also Supplementary Figure 1).

Table 1
Demographic, genetic and clinical characteristics of T1D patients of the present study.

Patient	Gender	Age of disease onset	Actual age	Duration of disease	Associated autoimmune disorders	*PTPN22* genotype
1	M	2.6	17	14.4	ATD;CD;AG	1858C/1858T
2	M	14	27	13	ATD	1858C/1858T
3	M	4.9	17.6	12.7	ATD;CD	1858C/1858T
4	F	12.4	15.8	3.4	ATD	1858C/1858T
5	M	4	20.4	16.4	ATD	1858C/1858T
6	M	1	16	15		1858C/1858T
7	M	12.7	15.6	2.9		1858C/1858T
8	F	7.6	9.11	1.51		1858C/1858T
9	M	11.1	12.3	1.2		1858C/1858T
10	F	7.1	10.7	3.6		1858C/1858T
11	F	7	20	13	ATD	1858C/1858T
12	F	10	24	14		1858C/1858T
13	F	14	28	14		1858C/1858T
14	M	7.1	13.1	6		1858C/1858T
15	M	4.11	6.11	2		1858C/1858T
16	M	10.1	13.9	3.8		1858C/1858T
17	F	4.5	19	14.5	ATD	1858C/1858C
18	F	3.2	25	21.8	ATD;V;IDA;AG	1858C/1858C
19	F	4	17	13	ATD	1858C/1858C
20	F	4.6	22	17.4	ATD;AG	1858C/1858C
21	M	6.2	8	1.8		1858C/1858C
22	M	8	11.9	3.9		1858C/1858C

V, Vitiligo; IDA, Iron-deficiency Anemia. Age is expressed in “years, months”.

C1858T PTPN22 gene variant down-modulation in human peripheral blood mononuclear cells

Study population

The study population included 22 T1D patients from the Endocrinology Division at Bambino Gesu` Children's Hospital. Of the total number of patients, 16 were heterozygous carriers of the C1858T *PTPN22* variant (HET), and 6 non-carriers (WT) (for detection method of the *PTPN22* C1858T variant see S4, Supplementary materials). All patients were recruited during long-term disease. The mean actual age of the T1D patients who were non-carriers of the C1858T *PTPN22* variant was 17.15 years (age range 8-25; 4 females, 2 males,) (Table 1). The mean age at disease onset was 5.08 years (age-range 3.2-8), and the mean duration of disease was 12.07 years (age range 1.8-21.8). The mean actual age of T1D patients who were carriers of the C1858T polymorphism was 16.66 years (age range 6.11-28; 6 females, 10 males) (Table 1). The mean age at onset of disease was 8.11 years (age range 1-12.7), and the mean disease duration was 8.56 years (age range 1.2-16.4). Diabetics’ demographic and clinical characteristics are shown in Table 1.

The patients' sera were assayed for GADA (isoform 65), IA2 antibodies (Abs) and IAA by radioimmunoassay, for Abs to thyroglobulin (Tg), thyroperoxidase (TPO) and transglutaminase (tTGA) by chemiluminescence (ADVIA Centaur analyzer, Siemens Healthcare, Germany) and to parietal cells (PCA) and the adrenal cortex by indirect immunofluorescence (IFL).

The T1D patients presented associated autoimmune disorders (Table 1). In addition to diabetes, 9 patients developed Hashimoto's thyroiditis (APS3v), confirmed by the presence of circulating Tg and TPO Abs and by an echography pattern of diffuse hypoechogenicity. Three of these also presented autoimmune gastritis (AG) confirmed by the presence of PCA, two were affected by celiac disease (CD) confirmed by the presence of tTGA and anti-gliadin Abs, and one patient also developed vitiligo (V), AG, iron-deficiency anemia (IDA) and autoimmune thyroid disease (ATD).

All recruited patients were unrelated. All subjects entered the study after written informed consent was obtained. The investigation was approved by the local Institutional Review Board (IRB) of Bambino Gesu` Children's Hospital, which regulates human samples usage for experimental studies (Study protocol no. 1385_OPBG_2017); all procedures followed were in accordance with institutional guidelines. The informed was obtained from the next of kin in case of children. Consent on behalf of children was written. Participant consent was recorded using a paper-based inventory system. The IRB approved the consent procedure.

Custom liposome transfection protocol

Cryopreserved T1D PBMCs (S5, Supplementary materials) were thawed, washed in complete RPMI 1640 medium (EuroClone, Pero (Milan), Italy) additioned with 10% fetal bovine serum (FBS, GE Healthcare Life Sciences, UT, USA) and L-glutamine (2 mM) (EuroClone). Cells were then cultured at 1.5×10^6 per well in 48-well plates (Falcon, Corning, NY, USA) in 250 μl of FBS-free RPMI 1640 medium containing L-glutamine (2 mM) and treated with different doses of Lipo/siRNA complexes (20, 60, 80, 100 pmol of siRNA). After an overnight (O/N) transfection, cells were washed by 1200 rpm centrifugation for 5 min. The cells were subsequently replated in 48-well plates (flat bottom) in 250 μl of complete RPMI medium.

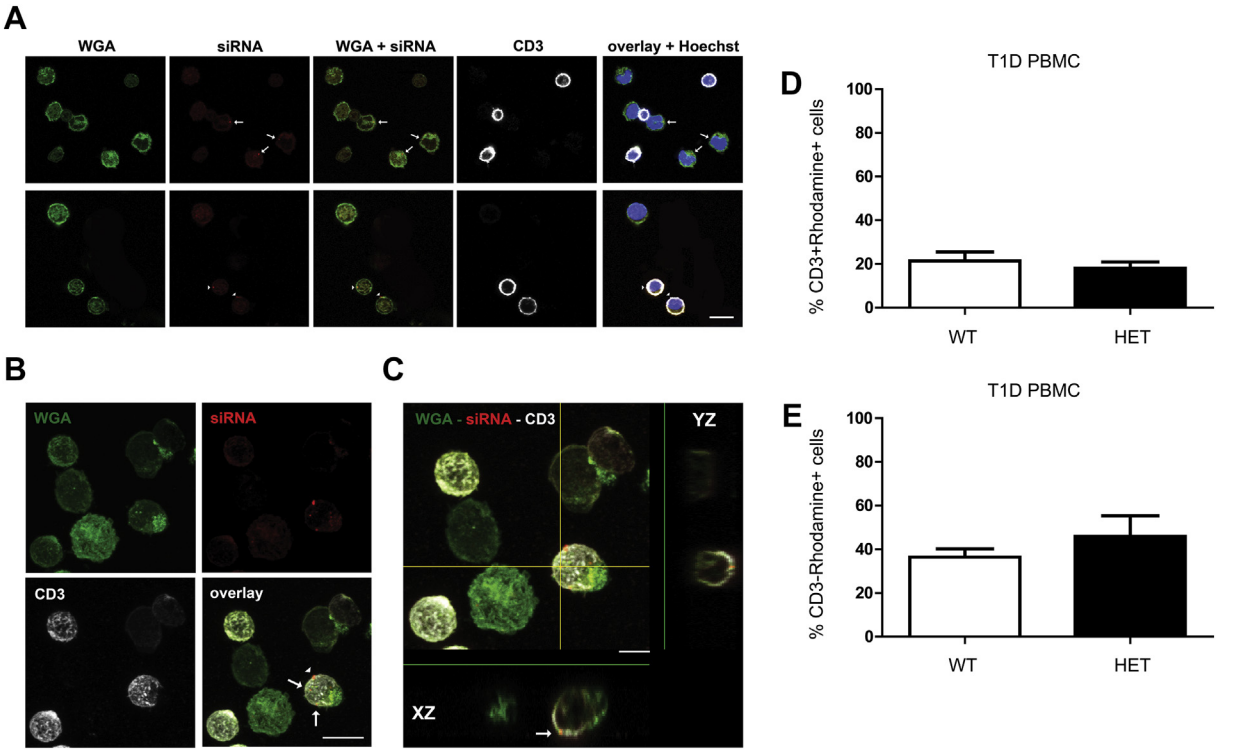

Figure 1. Immunofluorescence evaluation of Lipo/siRNA internalization in T1D PBMC. (**A**) Images depict lipoplexes (siRNA indicated by red dots and white arrows) inside $CD3^-$ and (**B**) $CD3^+$ (white) cells among PBMC of T1D patients already after 4.5 h of treatment (100 pmol). WGA (green) was used for cell membrane and Hoechst dye (blue) for cells nuclei. Bar: 20 μm. (**C**) Confocal Z reconstructions depict lipoplexes (indicated by red dots) inside the cytoplasm of $CD3^+$ lymphocytes. Bar: 10 μm. (**D**) Percentage of $siRNA^+$ cells ($Rhodamine^+$ cells) among wild-type (WT) and heterozygous C1858T *PTPN22* (HET) $CD3^+$ and (**E**) $CD3^-$ cells.

Cells were further incubated for 24 and 48 h at 37° in a 5% CO_2 humidified atmosphere.

RNA extraction, quantitative real Time-PCR (rtq_PCR), confocal microscopy analysis, toxicity and functional assay

Protocol details are found in the S6-S10 sections of the Supplementary materials.

Results

Evaluation of size and polydispersion of liposomes and lipoplexes by DLS measurements

DLS experiments on the liposome formulation of DMPC/2 and on lipoplexes (DMPC/2/siRNA or Lipo/siRNA) were performed as previously reported.[11] The investigations on the liposomes formulation confirmed what we already described: liposome formulation of DMPC/2 shows a single narrow population centered at about 40 nm after 9 h from the extrusion, whereas, after 72 h from their formulation, liposomes increase significantly in dimensions.[11]

Lipoplexes composed of the siRNA against the variant *PTPN22* and the DMPC/2 liposomes show a behavior similar to that observed for lipoplexes of wild type siRNA.[11] Indeed, also in the published case study, lipoplexes dimensions are not heavily affected by the siRNA presence, the dimensions being around 70 nm in diameter. In addition, the lipoplexes DMPC/2/siRNA appear only slightly increased up to ~90 nm with time, thus suggesting that lipoplexes are rather stable.

CD examination of the siRNA conformational stability in lipoplexes

Investigations of the lipoplexes composed of the siRNA against the variant *PTPN22* and DMPC/2 liposomes were performed following the same approach previously described.[11] Also in this case, at each measurement, the CD spectrum of siRNA in lipoplexes (DMPC/2/siRNA), was similar to that of siRNA that remains free in buffer. The bands of lipoplexes were less intense than those of free siRNA. These observations indicate that the combination of liposomes with siRNA does not affect significantly the conformational stability of siRNA designed against the variant *PTPN22*. In addition, the absence of marked variations in the CD spectrum over a 72 h period is an indirect confirmation of lipoplexes stability.

Lipo/siRNA SNP_T lipoplexes are effectively internalized in T1D PBMC

Internalization of rhodamine-conjugated Lipo/siRNA complexes (100 pmol of siRNA) was visualized in T1D PBMC (either wild type or heterozygous C1858T) following 4.5 h of incubation (Figure 1, *A* and *B*; red dots/white arrows indicate lipoplexes; green WGA and blue Hoechst indicate membrane

and nuclei respectively). The study of the projections on the X- and Y-axis of the Z-reconstructions of confocal single optical sections (Figure 1, *C*) allowed clear detection of lipoplexes beneath the cell membrane. Internalization of siRNA molecules inside T1D PBMC was confirmed by the observation relative to rhodamine positivity into these cells. This observation indicates lipoplexes efficacy as siRNA system of administration also to T1D PBMC, as previously observed for Jurkat T cells and healthy donor PBMC.[11] This result was reported in both wild-type (WT) and C1858T *PTPN22* heterozygous (HET) T1D PBMC (Figure 1, *D* and *E*).

The internalization was confirmed specifically in both $CD3^+$ (white) and $CD3^-$ T lymphocytes (Figure 1, *A*, *B* and *C*). Of note, no difference was observed regarding lipoplexes internalization efficacy between the $CD3^+$ (Figure 1, *D*) or $CD3^-$ (Figure 1, *E*) cells when analyzing wild-type versus (vs) C1858T *PTPN22* heterozygous T1D PBMC (Figure 1, *D* and *E*).

Lipo/siRNA SNP_T lipoplexes are not toxic to T1D PBMC

Treatment with rhodamine-marked lipoplexes (20, 60, 80 and 100 pmol of siRNA) did not reveal evidence of toxicity on PBMC, by means of quality and quantity of cell pellet and protein concentration analyzed at each experimental timeline.

T1D PBMC treated with different doses of rhodamine-conjugated lipoplexes for 4.5 h retained proper morphology of the cell membrane (marked in green) and nuclei (marked in blue) as observed by immunofluorescence analysis (Supplementary Figure 2), suggesting the absence of damage or apoptosis respectively (Supplementary Figure 2).

T1D PBMC, treated as above and analyzed by flow-cytometry, revealed high percentage of $rhodamine^+$ cells implying relevant transfection efficacy and internalization and, at the same time, showed low percentage of dead cells ($Rhodamine^+DAPI^+$ cells) (Figure 2) indicative of low lipoplexes toxicity at this specific timing of the experimental procedure.

Lipo/siRNA SNP_T lipoplexes treatment downregulates PTPN22 mRNA

The mRNA obtained from PBMC derived from 16 heterozygous C1858T *PTPN22* patients and 6 wild-type *PTPN22* patients was analyzed by rtq-PCR after treating the cells with different doses of lipoplexes (20, 60, 80 and 100 pmol of siRNA) for 48 and 72 h. Either time point of the lipoplexes treatment led to a decrease in the target *PTPN22* mRNA levels in 13 out of 16 heterozygous patients (Figures 3 and 4), while it did not affect the mRNA levels in the wild-type patients (Figure 3). These results indicate valuable efficacy of the lipoplexes under study to specifically downregulate variant T1858 *PTPN22* mRNA.

To ascertain lipoplexes variant specificity, we designed a second set of primers aimed to detect T1858 variant mRNA solely. We first validated these primers performing the rtq-PCR on PBMC derived from wild-type *PTPN22* T1D patients using both set of primers, the new specific one and the first one able to recognize all target gene mRNA. The result of this

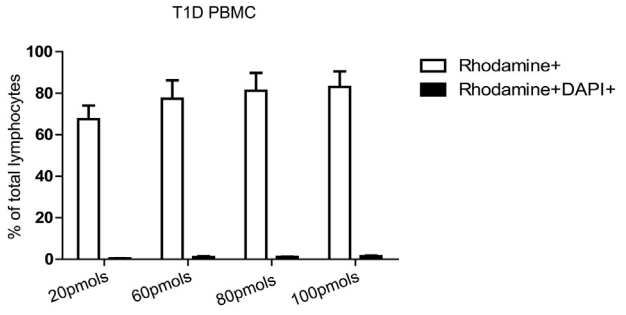

Figure 2. Evaluation of Lipo/siRNA toxicity on T1D PBMC. Flow cytometry analysis of T1D PBMC treated for 4.5 h with rhodamine-conjugated lipoplexes (100 pmol). Percentage of transfected lymphocytes (i.e. $Rhodamine^+$ elements) and relative percentage of dead cells ($Rhodamine^+DAPI^+$ cells).

validation showed the inability of the specific set to detect wild-type *PTPN22* mRNA where the T1858 SNP is not present (Figure 5, *A*). Subsequently, these primers were tested on the mRNA of heterozygous C1858T *PTPN22* T1D PBMC treated as above described for 72 h. In this specific experiment, the new primers clearly revealed the presence of the variant mRNA and reported its decrease upon lipoplexes treatment (Figure 5, *B* and *C*).

Lipo/siRNA SNP_T lipoplexes efficacy toward Lyp biological activity

Autoimmune disease associated R620W Lyp is a gain-of-function enzyme variant,[13] thus with higher phosphatase activity. We confirmed in our set of T1D patients data from literature[17] showing a decreased secretion of interleukin 2 (IL-2) by heterozygous C1858T *PTPN22* PBMC in comparison to wild-type *PTPN22* PBMC after anti-CD3/CD28 beads stimulus (Supplementary Figure 3). This significantly diverse response to TCR engagement was observed in all the activating conditions used (bead to cell ratios 1:1; 1:3,3; 1:10) (Supplementary Figure 3).

After TCR engagement, an increased IL-2 concentration upon lipoplexes treatment in respect to untreated cells (RPMI) was observed in heterozygous C1858T *PTPN22* T1D PBMC in comparison to wild-type *PTPN22* T1D PBMC (Figure 6). The same result was obtained and more evident using a suboptimal condition for stimulation with anti-CD3/CD28 beads (Supplementary Figure 4). This observation implies that the 'gain of function' effect of Lyp R620W on TCR signaling[13] can be rescued following treatment with lipoplexes. As a final consequence, this mode of action could restore normal Lyp regulatory performance.

Discussion

Metanalysis investigations come in support of the fact that in the Caucasian population *PTPN22* variant is a remarkable risk factor for T1D.[18] The impact of the C1858T *PTPN22* mutation in disease variability in European and American populations has been evaluated by unraveling its association with age of onset, autoantibodies levels, β cell residual activity and patients' metabolic control.[7]

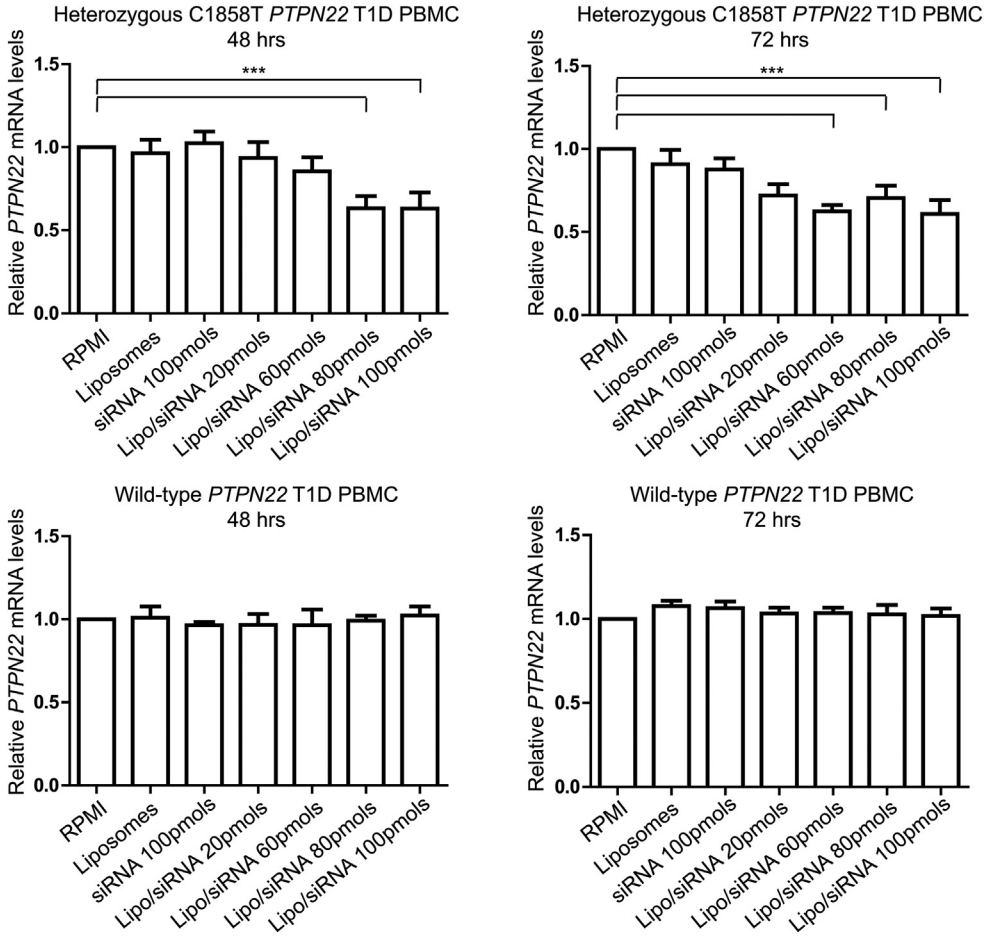

Figure 3. Assessment of Lipo/siRNA efficacy on target mRNA. (**A**) Decrease of target *PTPN22* mRNA in T1D PBMC analyzed by rqt-PCR upon 48 h of lipoplexes treatment. (**B**) Similar inhibition after 72 h was achieved. Statistical test was performed analyzing 13 responder heterozygous C1858T *PTPN22* patients. *** indicates one-way analysis of variance *P* value = 0.003 for the 48 h data and *P* = 0.002 for the 72 h data. (**C**) Target mRNA levels related to the 6 wild-type *PTPN22* patients upon 48 h and (**D**) 72 h of treatment.

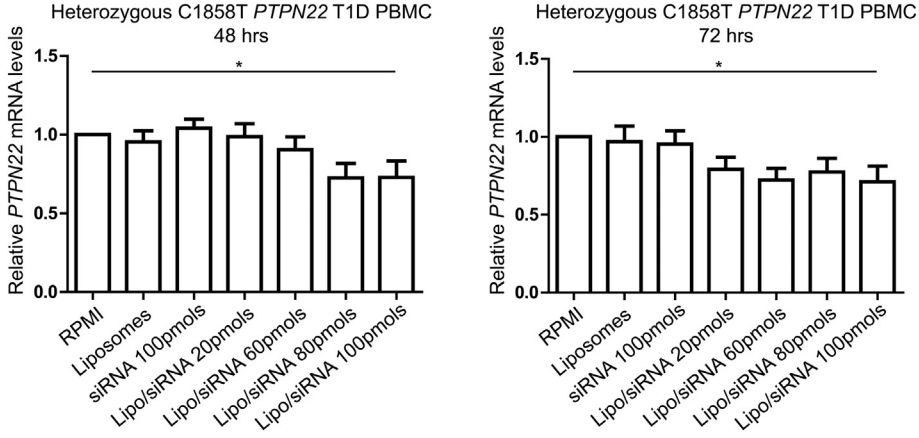

Figure 4. Overall analysis of Lipo/siRNA efficacy on target mRNA. (**A**) Target mRNA analysis deriving from all 16 heterozygous C1858T *PTPN22* patients tested including 3 non-responders at 48 and (**B**) 72 h after the indicated treatments. * indicates one-way analysis of variance *P* value = 0.0126 at 48 h and *P* value = 0.0401 at 72 h.

Andersen et al (2013)[19] emphasize a correlation between the presence of the variant with an earlier disease onset, or, a more rapid decline of the β cell reservoir upon the initial autoimmune attack. Further studies suggest that the variant can influence the progression from preclinical to overt disease in subjects with circulating islet cell autoantibodies.[20,21] Additionally, the variant allele correlated with worse metabolic control in long-term diabetes.[22] This led to hypothesize that carriers of the variant experienced a more

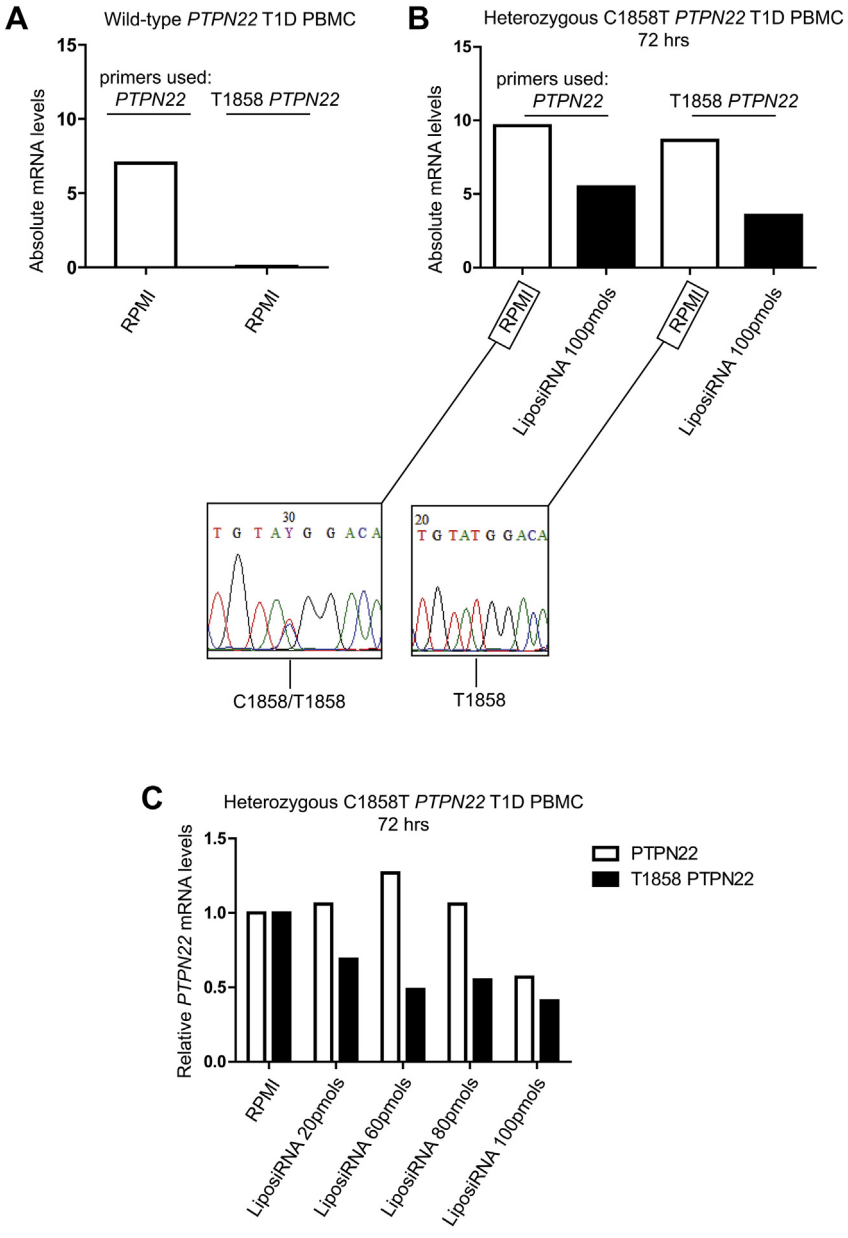

Figure 5. Evidence for Lipo/siRNA specificity toward T1858 *PTPN22* mRNA. (**A**) Rtq-PCR analysis of mRNA from wild-type *PTPN22* T1D PBMC using two different sets of primers to recognize whole target *PTPN22* gene or T1858 *PTPN22* mRNA. (**B**) Rtq-PCR analysis of mRNA from heterozygous C1858T *PTPN22* PBMC treated with lipoplexes at the indicated dose for 72 h, with the same two sets of primers as above described. Electropherograms show both C1858 and T1858 alleles in rtq-PCR products from untreated PBMC (RPMI) using primers that detect whole target mRNA, while the sole T1858 SNP is observed when using variant-specific set of primers. (**C**) Target mRNA detection of either set of primers in a single representative experiment. Primers used are indicated in the histogram legend.

destructive β cell damage and maintained significantly lower c-peptide levels compared to C1858 homozygotes within the first year. In this regard, among patients recruited in our Institution, in a group of 28 variant *PTPN22* heterozygous and 39 wild type T1D patients, the average age of disease onset was confirmed to be significantly lower in heterozygous individuals, particularly in male patients (Supplementary Figure 5, *A*). Moreover, a worse metabolic control was observed in heterozygous patients since higher levels of HbA1c and lower c-peptide were detected in long-term disease (Supplementary Figure 5, *B* and *C*).

In the light of the foregoing, Lyp selective high affinity noncompetitive inhibitors were designed and showed activity in primary T cells[23–25] as a potentially valuable approach in autoimmunity.

As reported in the Introduction, trying to evaluate the feasibility of an innovative immunotherapeutic strategy, we recently demonstrated the opportunity to down-regulating wild type *PTPN22* gene expression in Jurkat human T lymphoblastoid cells and in PBMC of healthy individuals through delivering originally designed siRNA duplexes by liposomal carriers. The

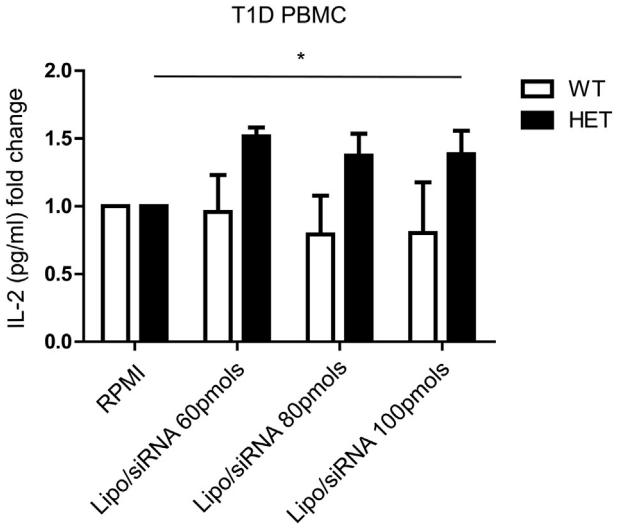

Figure 6. IL-2 detection in cultured supernatants of transfected then anti-CD3/CD28 stimulated PBMC. IL-2 secretion upon 20 h of anti-CD3/CD28 activation (bead to cell ratio 1:10) in culture supernatants of T1D *PTPN22* WT and C1858T *PTPN22* HET PBMC transfected O/N with lipoplexes at the indicated doses. * indicates one-way analysis of variance *P* value = 0.0491 for the HET PBMC. IL-2 amount was normalized for each sample to the total protein load (assayed by Pierce™ Thermo Scientific BCA (Rockford, IL) colorimetric protein kit).

biological effect of *PTPN22* down-regulation was functionally confirmed.[11]

In this manuscript, in order to evaluate the use of lipoplexes for a novel 'targeted' approach of immunotherapy against the variant *PTPN22* allele, we demonstrated the possibility to deliver efficiently siRNAs by the same cationic liposomes into PBMC of wild type as well as of heterozygous T1D patients. Confocal microscopy analysis observed that lipoplexes were depicted in both CD3+ and $CD3^-$ cells. These results suggest that new therapy effect is not exclusive to T lymphocytes.[11] Potentially, in translational studies that aim to target the variant *PTPN22* allele, the ideal lipoplexes treatment will concern its modulation to rescue the net normal activity of the allele in any immunocyte, where based on the existing literature, opposite effects could be variant-induced i.e. gain or loss of function.[12,16] As already pointed out in our previous manuscript,[11] in developing the novel targeted therapy, whenever specific T cell delivery would be necessary, functionalizing lipoplexes with FDA authorized anti-CD3 mAbs may be required in the future (rev in[11]).

It is of upmost importance to verify safety and efficacy of lipoplexes prior to undertaking any translational therapeutic approach in patients.[11] In this regard, liposomes were approved for human use by FDA since of low toxicity in humans, therefore already utilized in clinical trials (rev in[11]). As previously shown in Jurkat T and PBMC cultures from healthy donors,[11] even cryopreserved PBMC from T1D patients did not reveal signs of toxicity. It is however important to remark that before undertaking human studies, the treatment biodistribution efficacy and safety should be ascertained in preclinical experimental animal investigations, i.e. the NOD (nonobese diabetic) mouse.[26]

Liposome/siRNA complexes treatment revealed a significant decrease in target variant *PTPN22* mRNA by quantitative Real-Time PCR in a total of 13 out of 16 heterozygous patients (approximately 81.2% of analyzed samples) while there was no effect on wild-type patients as expected. Interestingly, results were confirmed by the analysis with both primers detecting content of target *PTPN22* mRNA or solely T1858 variant mRNA.

In preliminary experiments, we tried to functionally assess the biological effects of variant *PTPN22* down-regulation since it would restore the net activity of the wild-type allele. In this regard, it is known from literature that the disease-associated variant affects T cell activation more promptly than LYP-Arg620.[13] Indeed, we confirmed reduced IL-2 production in primary T cells from PBMC of heterozygous T1D patients compared with those of wild type T1D patients following TCR engagement (Supplementary Figure 3). In our experiments, lipoplexes treatment in a dose ranging from 60 to 100 pmol of siRNA restored in heterozygous, compared to wild-type T1D patients, IL-2 levels of secretion upon 20 h of anti-CD3/CD28 PBMC stimulation. This was further verified using Lipo/siRNA (100 pmol) in a prolonged time course of anti-CD3/CD8 stimulation already experienced ideal when exploiting immunomodulation.[11]

While unraveling the potential utility of targeting C1858T *PTPN22* in autoimmunity, we need to consider that, in addition to T1D, the variant was also found in association with other organ and non-organ specific autoimmune disorders in Caucasians.[27–32]

In the light of the foregoing, we may envisage that lipoplexes targeting the *PTPN22* variant can find widespread applicability. For personalized treatment, functionalization of lipoplexes with monoclonal antibodies generated against peculiar immunotypes i.e. anti-CD20 Abs to target B lymphocytes may additionally be requested while sparing tolerogenic B regulatory cells.[33] Definitively, deepening the knowledge in the etiopathogenesis of different autoimmune conditions where peculiar immunotypes are predominantly involved, would help to appropriately direct the novel lipoplexes based-subsidiary immunotherapeutic treatment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2018.11.001.

References

1. Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. *Lancet* 2014;**383**:69-82, https://doi.org/10.1016/S0140-6736(13)60591-7.
2. Krzewska A, Ben-Skowronek I. Effect of associated autoimmune diseases on type 1 diabetes mellitus incidence and metabolic control in children and adolescents. *Biomed Res Int* 2016;**2016**6219730, https://doi.org/10.1155/2016/6219730.
3. Buscemi S, Massenti FM, Vasto S, Galvano F, Buscemi C, Corleo D, et al. Association of obesity and diabetes with thyroid nodules. *Endocrine* 2018;**60**:339-47, https://doi.org/10.1007/s12020-017-1394-2.
4. Woittiez NJ, Roep BO. Impact of disease heterogeneity on treatment efficacy of immunotherapy in type 1 diabetes: different shades of gray. *Immunotherapy* 2015;**7**:163-74, https://doi.org/10.2217/imt.14.104.

5. Atkinson MA, Ogle GD. Improving diabetes care in resource-poor countries: challenges and opportunities. *Lancet Diabetes Endocrinol* 2013;**1**:268-70, https://doi.org/10.1016/S2213-8587(13)70172-4.
6. Wiebolt J, Koeleman BP, van Haeften TW. Endocrine autoimmune disease: genetics become complex. *Clin Invest* 2010;**40**:1144-55, https://doi.org/10.1111/j.1365-2362.2010.02366.x.
7. Gianchecchi E, Palombi M, Fierabracci A. The putative role of the C1858T polymorphism of protein tyrosine phosphatase *PTPN22* gene in autoimmunity. *Autoimmun Rev* 2013;**12**:717-25, https://doi.org/10.1016/j.autrev.2012.12.003.
8. Zhang N, Huang W, Dong F, Liu Y, Zhang B, Jing L, et al. Insulin gene VNTR polymorphisms -2221MspI and -23HphI are associated with type 1 diabetes and latent autoimmune diabetes in adults: a meta-analysis. *Acta Diabetol* 2015;**52**:1143-55, https://doi.org/10.1007/s00592-015-0805-1.
9. Wang K, Zhu Q, Lu Y, Lu H, Zhang F, Wang X, et al. CTLA-4+49 G/a polymorphism confers autoimmune disease risk: an updated meta-analysis. *Genet Test Mol Biomarkers* 2017;**21**:222-7, https://doi.org/10.1089/gtmb.2016.0335.
10. Zhang J, Chen Z, Zhou Z, Yang P, Wang CY. Sumoylation modulates the susceptibility to type 1 diabetes. *Adv Exp Med Biol* 2017;**963**:299-322, https://doi.org/10.1007/978-3-319-50044-7_18.
11. Perri V, Pellegrino M, Ceccacci F, Scipioni A, Petrini S, Gianchecchi E, et al. Use of short interfering RNA delivered by cationic liposomes to enable efficient down-regulation of PTPN22 gene in human T lymphocytes. *PLoS One* 2017;**12**e0175784, https://doi.org/10.1371/journal.pone.0175784.
12. Maine CJ, Hamilton-Williams EE, Cheung J, Stanford SM, Bottini N, Wicker LS, et al. PTPN22 alters the development of regulatory T cells in the thymus. *J Immunol* 2012;**188**:5267-75, https://doi.org/10.4049/jimmunol.1200150.
13. Vang T, Congia M, Macis MD, Musumeci L, OrruÂ V, Zavattari P, et al. Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. *Nat Genet* 2005;**37**:1317-9, https://doi.org/10.1038/ng1673.
14. Gianchecchi E, Crinò A, Giorda E, Luciano R, Perri V, Lorusso A, et al. Altered B cell homeostasis and toll-like receptor 9-driven response in type 1 diabetes carriers of the C1858T PTPN22 allelic variant: implications in the disease pathogenesis. *PLoS ONE* 2014;**9**e110755, https://doi.org/10.1371/journal.pone.0110755.
15. Li M, Beauchemin H, Popovic N, Peterson A, d'Hennezel E, Piccirillo CA, et al. The common, autoimmunity-predisposing 620Arg>Trp variant of PTPN22 modulates macrophage function and morphology. *J Autoimmun* 2017;**79**:74-83, https://doi.org/10.1016/j.jaut.2017.01.009.
16. Zhang J, Zahir N, Jiang Q, Miliotis H, Heyraud S, Meng X, et al. The autoimmune disease-associated PTPN22 variant promotes calpain-mediated Lyp/Pep degradation associated with lymphocyte and dendritic cell hyperresponsiveness. *Nat Genet* 2011;**43**:902-7, https://doi.org/10.1038/ng.904.
17. Aleandri S, Bonicelli MG, Bordi F, Casciari S, Diociaiuti, Giansanti L, et al. How stereochemistry affects the physicochemical features of gemini surfactant based cationic liposomes. *Soft Matter* 2012;**8**:5904-15.
18. Xuan C, Lun LM, Zhao JX, Wang HW, Zhu BZ, Yu S, et al. PTPN22 gene polymorphism (C1858T) is associated with susceptibility to type 1 diabetes: a meta-analysis of 19,495 cases and 25,341 controls. *Ann Hum Genet* 2013;**77**:191-203, https://doi.org/10.1111/ahg.12016.
19. Andersen ML, Rasmussen MA, Pörksen S, Svensson J, Vikre-Jørgensen J, Thomsen J, et al. Complex multi-block analysis identifies new immunologic and genetic disease progression patterns associated with the residual β-cell function 1 year after diagnosis of type 1 diabetes. *PLoS One* 2013;**8**e64632, https://doi.org/10.1371/journal.pone.0064632.
20. Hermann R, Lipponen K, Kiviniemi M, Kakko T, Veijola R, Simell O, et al. Lymphoid tyrosine phosphatase (LYP/PTPN22) Arg620Trp variant regulates insulin autoimmunity and progression to type 1 diabetes. *Diabetologia* 2006;**49**:1198-208.
21. Kumar N, Kaur G, Kanga U, Tandon N, Caillat-Zucman S, Mehra NK. Association of PTPN22+1858C/T polymorphism with type 1 diabetes in the North Indian population. *Immunogenet* 2014;**41**:318-23, https://doi.org/10.1111/iji.12129.
22. Petrone A, Spoletini M, Zampetti S, Capizzi M, Zavarella S, Osborn J, et al. The PTPN22 1858T gene variant in type 1 diabetes is associated with reduced residual beta-cell function and worse metabolic control. *Diabetes Care* 2008;**31**:1214-8, https://doi.org/10.2337/dc07-1158.
23. Stanford SM, Krishnamurthy D, Falk MD, Messina R, Debnath B, Li S, et al. Discovery of a novel series of inhibitors of lymphoid tyrosine phosphatase with activity in human T cells. *J Med Chem* 2011;**54**:1640-54, https://doi.org/10.1021/jm101202j.
24. Vang T, Xie Y, Liu WH, Vidović D, Liu Y, Wu S, et al. Inhibition of lymphoid tyrosine phosphatase by benzofuran salicylic acids. *J Med Chem* 2011;**54**:562-71, https://doi.org/10.1021/jm101004d.
25. He Y, Liu S, Menon A, Stanford S, Oppong E, Gunawan AM, et al. A potent and selective small-molecule inhibitor for the lymphoid-specific tyrosine phosphatase (LYP), a target associated with autoimmune diseases. *J Med Chem* 2013;**56**:4990-5008, https://doi.org/10.1021/jm400248c.
26. Fierabracci A. Peptide immunotherapies in type 1 diabetes: lessons from animal models. *Curr Med Chem* 2011;**18**:577-86.
27. Vandiedonck C, Capdevielle C, Giraud M, Krumeich S, Jais JP, Eymard B, et al. Association of the PTPN22*R620W polymorphism with autoimmune myasthenia gravis. *Ann Neurol* 2006;**59**:404-7.
28. Sadovnick AD. Genetic background of multiple sclerosis. *Autoimmun Rev* 2012;**11**:163-6, https://doi.org/10.1016/j.autrev.2011.05.007.
29. Jagiello P, Aries P, Arning L, Wagenleiter SE, Csernok E, Hellmich B, et al. The PTPN22 620W allele is a risk factor for Wegener's granulomatosis. *Arthritis Rheum* 2005;**52**:4039-43.
30. Orozco G, Sánchez E, González-Gay MA, López-Nevot MA, Torres B, Cáliz R, et al. Association of a functional single-nucleotide polymorphism of PTPN22, encoding lymphoid protein phosphatase, with rheumatoid arthritis and systemic lupus erythematosus. *Arthritis Rheum* 2005;**52**:219-24.
31. Hinks A, Barton A, John S, Bruce I, Hawkins C, Griffiths CE, et al. Association between the PTPN22 gene and rheumatoid arthritis and juvenile idiopathic arthritis in a UK population: further support that PTPN22 is an autoimmunity gene. *Arthritis Rheum* 2005;**52**:1694-9.
32. Gourh P, Tan FK, Assassi S, Ahn CW, McNearney TA, Fischbach M, et al. Association of the PTPN22 R620W polymorphism with anti-topoisomerase I- and anticentromere antibody-positive systemic sclerosis. *Arthritis Rheum* 2006;**54**:3945-53.
33. Giannoukakis N, Trucco MA. A role for tolerogenic dendritic cell-induced B-regulatory cells in type 1 diabetes mellitus. *Curr Opin Endocrinol Diabetes Obes* 2012;**19**:279-87.